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13. ABSTRACT (Maximum 200 words)
The Systemic Inflammatory Response Syndrome (SIRS) that frequently occurs post-injury, is a major cause of post-trauma mortality and is characterized by both immunosuppression and cytokine aberrations. This research focuses on identifying the relative contributions of T cell and monocyte (MØ) aberrations to SIRS. In the first half of the contract, we have shown that MØ TNF_α production (a major contributor to SIRS) is aberrantly increased in trauma patients by: 1.Failure to rapidly degrade TNF_α mRNA leading to prolonged TNF_α protein production; 2.Insensitivity to normal downregulation by prostaglandin E_2 and TGF_β; 3.Increased sensitivity of MØ to TNF_α induction by LTB_4; 4.Increased MØ autocrine stimulation by TNF_α due to stimulation through unshed TNFR; 5.Decreased production of the TNF_α immunoregulatory cytokine IL-10 by patients' MØ and T lymphocytes; 6.Decreased T lymphocyte production of IFN_γ resulting in MØ TNF_α induction by trauma generated mediators in the absence of T lymphokines; and 7.Loss of T lymphocyte function due partially to depression of MØ co-activators such as IL-12. We have also found that excessive monokine production can be downregulated by addition of regulatory mediators such as IL-10 and IL-4. Finally, we have shown that post-trauma immunosuppressive levels of PGE_2 can be reduced without increasing MØ TNF_α levels by treatment with both cyclo-oxygenase and lipoxygenase inhibitors.

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FOREWORD

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Carol P. Williamson 11/14/94

PI Signature Date

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INTRODUCTION:

The Systemic Inflammatory Response Syndrome (SIRS) frequently occurs post-injury and is characterized by immunosuppression and cytokine aberrations. SIRS is a major cause of morbidity and mortality, as well as prolonged hospital stays in trauma patients. Combat conditions may not allow post-injury prevention of bacterial contamination during the post-trauma period of immunosuppression. Consequently, mechanisms of ameliorating immunosuppression and subsequent cytokine alterations are a major priority in combat casualty care research. Research supported by this contract has focused on defining post-trauma patients' monocyte (MØ) functions. This monitoring of patients' MØ dysfunctions requires evaluation, implementation, and adaptation of new assays and techniques for assessing patients' MØ and T cell functions. The relative contribution to SIRS of the identified post-trauma alteration is delineated. Targeted therapeutic treatments, such as addition of depressed cytokines or stimulation with different activators, are evaluated for restoring appropriate MØ and/or T cell function in vitro to trauma patients' cells. We are evaluating potential therapeutic treatment for their ability to restore appropriate function at the protein and molecular levels.

MATERIALS AND METHODS:

Patient population:

Patients with mechanical trauma (injury severity score > 25) or thermal trauma (>20% total body surface 2-3º burns) admitted to the University of Massachusetts Medical Center Trauma Unit or Burn Unit, Worcester, were included in the study. Normal controls were tested along with each patient. Volunteers from laboratory and hospital staff at the University of Massachusetts Medical Center served as normal controls. Informed consent was obtained from all patients and controls and the study was approved by the Institutional Review Board.

Monocyte separation and stimulation:

Monocytes from patients' and normals' blood were isolated from Ficoll-Hypague gradient-separated mononuclear cells (PBMC) by selective adherence as described previously (1). Briefly, nonadherent cells were removed after 1.5h of adherence to microexudate-treated plastic surface resulting in a >95% MØ purity as determined by fluorescein isothiocyanate (FITC) labelled OKM5 staining. $Fc_\gamma RI$ cross-linked populations were obtained by rosetting the MØ with human 0, Rh 0 $(D)^+$ erythrocytes, treated with anti-Rh 0 (D) human immunoglobulin as previously described (2,3). This rosetting technique provides cross-linking stimulation of the high-density $Fc_\gamma RI$ -bearing ($Fc_\gamma RI^+$) MØ subpopulations and yields an enriched $Fc_\gamma RI^+$ population (3,4). Three million MØ/well were cultured in 3.0 ml of RPMI 1640 medium, supplemented with 15% FBS, antibiotics, 4mM L-glutamine, 1mM pyruvate and 1% minimal essential medium (MEM). Endotoxin contamination was less than 15 pg/ml in the culture media and FBS and all media contained 100U/ml polymyxin B sulfate.

The unseparated MØ population and $Fc_\gamma RI^+$ MØ were stimulated with 20 μg/ml of the bacterial cell wall analogue, muramyl dipeptide (MDP) or a combination of 2 hours priming with 10U or 100U IFNγ /ml plus 20 μg/ml MDP. To determine the effects of different agents on cytokine production by MØ and/or to determine the mechanisms of altered cytokine production in trauma patients, different experiments were conducted in which those agents were added to the culture medium. TGF_β (2.4 or 4.8ng/ml), IL-10 (50U/ml), TNF_α (200U/ml), IL-4 (6ng/ml), PGE_2 (10^{-7}M), LTB4 (10^{-7}M), indomethacin (10^{-6}M), nordihydroguaiaretic acid (NDGA, 40 μM) were used in this manner. MØ supernates were collected after 16-18 h of stimulation and kept frozen at -80°C until different monocyte secreted factors were assayed. Adherent MØ were collected by EDTA treatment and scraping and recovered cells were kept frozen at $5x10^6$/ml concentration in PBS for further analysis.

Analysis of TNF-receptors of MØ:

Freshly isolated patients' and paired normals' nonadherent MØ, as well as overnight cultured MØ, both unstimulated and MDP or MDP+IFNγ stimulated, were analyzed for TNF receptor expression as described (5).

Mitogen assays:

PBMC ($2x10^5$ cells/200µl/well) were cultured in flatbottomed microtiter plates in presence or absence of PHA. The experiments were set up in duplicate. In one set, supernates were harvested after 30 hours and stored at -80°C until the day of assay for IL-10. In another set, cells were cultured for 72 hours for proliferation assays for [^{3}H]-thymidine incorporation at the last 18 hours of incubation. IL-12 was added in some experiments at the concentration of 100U/ml.

T cell purification and stimulation:

PBMC T cells were purified from PBMC as previously described (2,6). Briefly, normals' and patients' PBMC were depleted of MØ by selective adherence to microexudate-treated plastics surfaces. Nonadherent cells were rosetted with neuraminidase treated sheep red blood cells (SRBC). The SRBC-rosetted cells were >90% T cells with <1% contamination by B cells or monocytes, as determined by flow cytometric analysis. The purified T cells were cultured ($2x10^5$ cells/200µl/well) in flat bottomed microtiter plates in presence of immobilized anti-CD3 and anti-CD4. Mabs were immobilized onto plastic microtiter plates as described (7). In brief, anti-CD3 diluted in RPMI 1640 were placed (1.5 µg/50µl/well) in each of the wells of 96 well flat-bottomed microtiter plates and incubated at room temperature for 1.5 hr and then washed with PBS two times to remove nonadherent Mab. The process was repeated with anti-CD4 (1µg/50µl/well) . After 24 h of culture, 100 µl of supernates were harvested from each well and replenished with 100 µl of fresh medium and continued for proliferation for another 48 h in a [^{3}H] thymidine incorporation assay.

Assay for different MØ/T cell secreted factors:

TNF_α activity in MØ supernates (secreted TNF_α) and sonicated MØ lysates (cell-associated TNF_α) was measured in the L-M cell bioassay as previously described (3). Secreted

TNF_α was also measured by specific ELISA. IL-1 activity in the MØ culture supernates were measured by D10.G4.1 cell bioassay (8). The MØ supernates were assayed for IL-6 using the B9 cell bioassay as previously described (9,10) IL-6 levels in MØ culture supernates were also measured by a specific ELISA kit. IL-10 levels in the supernates of PBMC, MØ, and T cells were determined by a specific ELISA kit. TGF_β bioactivity in the MØ supernates was determined using the TGF_β -sensitive Mink Lung (Mv1Lu) connective tissue cell line (10,11). PGE_2 and IL-8 levels in the monocyte supernates were measured by respective specific ELISA kits. IFN_γ levels in the T cell supernates were assessed by specific ELISA.

Molecular Assays:

Preparation of Plasmid DNA:

Plasmid preparation was carried out as described (11).

Northern Blot Analysis:

Northern analysis of cellular RNA was carried out as described (11)

Quantitative Analysis of Cytokine Specific RNA:

Total cytoplasmic RNA was isolated from cultured cells using TRI-REAGENT according to manufacturer's instructions. Equivalent amounts of RNA were reverse transcribed. Resulting cDNA was used in competitive polymerase chain reactions (PCR) in which serial dilutions of appropriate CLONTECH MIMIC were used as competitors for cell derived specific cDNA. To standardize samples, competitive PCR was also carried out for G_3PDH again using the CLONTECH MIMIC system. All amplifications were carried out on a Perkin Elmer 480 Thermal Cycler. One cycle of 94°C for two minutes followed by three cycles of 94°C for one minute, one minute at 56°C, and two minutes at 72°C followed by twenty-seven cycles of one minute at 94°C, one minute at 60°C, and two minutes at 72°C followed by one cycle of 72°C for ten minutes were performed on all samples. PCR products were electrophoresed through 1.2% agarose TAE gels containing 0.1μg/ml ethidium bromide. The intensity of the resulting bands were determined through densitometric analyses and quantitation of original specific RNA was determined by comparison of target and MIMIC band intensities and were standardized by G_3PDH quantities.

RESULTS:

During the period 1/6/92 to 9/30/94, 46 patients were monitored, for a total of 206 post-injury time point samples. There were 24 thermally injured patients (>30% 3° burn) and 22 trauma patients (ISS >25) in this group. Altered mitogen responses in the patients' PBMC population, their MØ production of both cell-associated and secreted tumor necrosis factor (TNF_α), and their MØ levels of prostaglandin E_2 (PGE_2) were all routinely assessed in this study (Table 1). In addition, a number of other cytokine mediators and lymphocyte functions were assessed to further develop our profile of post-trauma dysfunctions and to delineate the mechanisms by which trauma can derange cytokine function leading to SIRS. As can be seen in Table 1, patients with significantly depressed (>35%) mitogen induced response (PHA) also had increased PGE_2 levels. Although elevated MØ TNF_α levels only occurred in such immunosuppressed patients, not all immunosuppressed patients had elevated MØ TNF_α production. These data may indicate that depressed mitogen responsiveness is occurring independently of increased TNF_α production and that SIRS may be separable from immunosuppression. Alternatively, immunosuppression with its resulting alterations in T cell lymphokine production and T cell function may become so severe as to allow the deregulation of monokine production which is manifested as SIRS.

In one set of experiments, we have concentrated on dissecting a number of mechanisms which contribute to post-trauma MØ overproduction of TNF_α, a major contributor to SIRS. These altered mechanisms could include: First, overstimulation of TNF_α mRNA by MØ due both to activation through normal quiescent pathways, such as crosslinking $Fc_\gamma RI$ receptors on the MØ surface, or simultaneous MØ stimulation by trauma generated mediators and bacterial products in the absence of appropriate T lymphokines. Second, loss or altered sensitivity to normal TNF_α regulatory pathways, such as PGE_2 and/or TGF_β downregulation. Third, increased sensitivity to autocrine stimulation (i.e. MØ produced TNF_α stimulates more TNF_α) due to altered downregulation of TNF receptors on the MØ surface.

In the second set of experiments, we have focused on altered T lymphocyte function both as a cause of increased susceptibility to bacterial infection and as a contributor to aberrant MØ

production of cytokines, such as IL-6 and TNF_α. This aberrant cytokine production may arise through the failure to produce regulatory lymphokines and/or through excessive bacterial stimulation of MØ caused by adequately controlled bacterial challenge. MØ function and T cell function are obviously intertwined, since inadequate MØ production of some monokines or co-stimulatory molecules leads to T cell anergy while failure of MØ to respond to T lymphokine regulation can be a result of MØ loss of receptors or MØ activation through alternative T lymphokine independent pathways. Consequently, we are examining not only T cell activity after injury, but also MØ response to T cell lymphokines, as well as MØ ability to activate T cells post-trauma. Concomitant to these experiments, we are evaluating the ability of various therapeutic treatments to ameliorate or reverse the T cell and MØ dysfunction we identify as a starting point for developing useful prophylactic therapy.

In the first set of experiments examining the mechanism behind excessive TNF_α production by MØ was examined. We have developed the Mimic PCR System to quantitate the levels of TNF_α mRNA produced in response to bacterial stimulation by trauma patients' MØ. As described in Methods and illustrated in Figure 1, the amount of TNF_α mRNA produced by patients' MØ, both isolation stimulated and stimulated by the gram positive bacterial cell wall analogue MDP, is compared to the same levels of mRNA produced by a simultaneously run normal's MØ. Any variation in original reverse transcripted cDNA loading amounts between normals' and patients' MØ samples are corrected for by assaying the concentrations of the housekeeping gene G_3PDH (Fig.1). We compared the amount of mRNA present after 3 hours of stimulation to the amount of bioactivity present after 16 hours of stimulation. As can be seen in Table 2, the amounts of mRNA produced by patients' MØ were always elevated when their TNF_α bioactivity was elevated. However, the level of increase in transcription of patients' TNF_α mRNA versus normals' was not directly related to the degree of excessive TNF_α bioactivity at 16 hours. One mechanism for regulation of TNF_α mRNA levels in normal individuals is through the inducing a TNF_α transcriptional inhibitor at the same time that TNF_α mRNA is induced. As a result, TNF_α mRNA levels are downregulated as this transcription inhibitor increases in

concentration (12). It was possible, therefore, that patients' MØ were not being stimulated to produce appropriate levels of a transcriptional inhibitor when they were induced for TNF_α mRNA production through normally quiescent pathways. In this case, failure to downregulate TNF_α mRNA transcription would result in excessive TNF_α protein production. To examine this possibility, we added the protein inhibitor, cycloheximide, to the normal and patient MØ cultures during TNF_α induction. The addition of cycloheximide (CHX) to normal MØ is known to superinduct TNF_α through suppression of transcriptional and translational protein inhibitors and consequent failure to limit TNF_α induction. TNF_α gene induction itself does not require new protein production (12). If the patients' MØ were producing excessive TNF_α mRNA and protein because no inhibitor was being induced, then CHX treated cultures should fail to be super-induced for TNF_α mRNA even though normals' MØ would show increased induction. As can be seen in Table 3, this was not the outcome. CHX treatment of both patients' and normals' MØ resulted in increased TNF_α mRNA. In fact, CHX treatment of patients' MØ resulted in even more massive induction of TNF_α mRNA (Table 3). Table 3 illustrates not only this massive super-induction of TNF_α mRNA in patients' MØ, but also demonstrates that aberrant MØ production of TNF_α mRNA increases over time post-injury and then returns to normal if the patient recovers. As can also be seen, those trauma patients' MØ which produce normal levels of TNF_α protein also have normal levels of TNF_α mRNA.

If the aberrantly high levels of both TNF_α mRNA and bioactivity produced by some patients' MØ is not a result of their loss of TNF_α mRNA inhibitors, then what other mechanisms can be invoked for these aberrant TNF_α levels? The CHX data showing massive super-induction of TNF_α in these patients' MØ provide a clue. Another mechanism to control TNF_α protein levels at the mRNA level is the extremely short half life of TNF_α mRNA (12). Because TNF_α mRNA is very unstable, rapid degradation of mRNA allows only a short translation period for TNF_α protein. Many cytokine inhibitors of TNF_α, such as IL-4 and IL-10, act by further decreasing the TNF_α mRNA stability, thereby preventing protein production. Other MØ stimulators, such as LPS, are known to increase TNF_α mRNA stability, thereby increasing the amounts of TNF_α

protein induced by other stimulators. Therefore, one mechanism by which trauma-generated mediators could increase TNF_α protein levels produced by patients' MØ is to increase the stability of the induced TNF_α mRNA.

To investigate this possibility, we examined the amount of TNF_α mRNA contained in patients' versus normals' MØ at 2 hours and 16-18 hours post-stimulation. Since we have already shown that patients have increased levels of mRNA at 2 hours as compared to normals, simply showing that the patients' MØ still had increased mRNA levels at 16-18 hours would not address a decrease in decay rate. To accurately compare the rate of TNF_α decay between patients and normals, we have reported the data as percent residual mRNA at 16 hours versus 2 hours. As illustrated in Table 4, these types of comparisons split the patients with increased MØ TNF_α bioactivity into two groups. One group (a minority) had increased TNF_α mRNA stability as well as increased TNF_α bioactivity (4a). MØ from the other group had increased TNF_α bioactivity and TNF_α mRNA, but normal TNF_α mRNA stability (4b). In this second group, the patients' MØ were <u>in vivo</u> activated to produce more TNF_α protein, but this protein was being downregulated in a normal manner. Consequently, truly excessive TNF_α protein levels would not be produced in response to an infectious challenge by these patients' MØ. However, MØ from the first group of patients (4a) was responding to bacterial challenge (MDP) by a prolonged production of TNF_α mRNA and protein which occurred because these patients' MØ failed to downregulate their TNF_α mRNA in a rapid fashion. Of five patients whose MØ exhibited increased mRNA stability, four went on to succumb from SIRS while the fifth had a prolonged and difficult clinical course and was clinically characterized as being in "septic shock". These data strongly support the hypothesis that trauma patients with excessive TNF_α production may have MØ which have been <u>in vivo</u> activated by pathways that circumvent normal TNF_α regulatory steps. One important implication of these findings is that only a subset of trauma patients with high TNF_α production will proceed to cytokine deregulation and SIRS. Identification of these patients by their MØ dysfunction could result in their segregation for more aggressive immunotherapy. In particular, cytokines like IL-4 and/or IL-10, which decrease MØ production of TNF_α by increasing the molecular regulation of

TNF_α mRNA, will be evaluated in this system to determine if they can reinstate TNF_α mRNA stability.

Other regulatory defects, besides prolonged TNF_α mRNA stability, are probably also involved in the excessive TNF_α production by trauma patients. We had previously shown that trauma patients' MØ were concomitantly producing high levels of PGE_2 and high levels of TNF_α proteins. This is an unexpected finding, since PGE_2 production by MØ normally occurs secondary to TNF_α protein production and acts to downregulate TNF_α at the transcription level by interfering with TNF_α gene induction. Our data showing concomitant production of TNF_α and PGE_2 in unstimulated patients' MØ led us to suggest that patients' MØ TNF_α production was PGE_2 insensitive. To test this hypothesis, we added exogenous PGE_2 to the patients' MØ cultures and examined subsequent production of both cell-associated and secreted TNF_α. This work has been published (13) and, therefore, will be only briefly summarized. These PGE_2 addition experiments demonstrated that patients' cell-associated TNF_α was highly insensitive to PGE_2 downregulation while secreted TNF_α levels were reduced by PGE_2 addition (Fig.2). These data suggested that post-trauma high PGE_2 levels may cause a redistribution of TNF_α from the secreted to the cell-associated forms. It is our hypothesis that the cell-associated form of TNF_α may be more pathological in that higher concentrations of TNF_α can be delivered by MØ bound directly to target cells because target cell adhesion molecules are also upregulated (for example, lung). It has already been demonstrated that cell-associated TNF_α is more cytolytic for tumor cells and the same high concentrations of TNF_α directly delivered by these MØ have been invoked to explain this result. When normal MØ are stimulated by crosslinking of the $Fc_\gamma RI$ receptors (i.e. CD64), they also produce greater levels of cell-associated TNF_α. We have previously shown trauma patients' MØ to express high levels of $Fc_\gamma RI$ and suggested that the increased circulating IgG levels known to typify these patients may also result in crosslinking and stimulation of the patients' MØ $Fc_\gamma RI$ inducing TNF_α. Normal MØ stimulated by crosslinking their $Fc_\gamma RI$ not only produce cell-associated TNF_α, but this TNF_α is also more resistant to PGE_2 downregulation (Fig.3). One

cause of increased cell-associated TNF_α in trauma patients may be increased PGE_2 levels and this cell-associated TNF_α is insensitive to PGE_2 downregulation.

We have also further explored the interactive effects of PGE_2 and LTB_4. Earlier experiments with exogenous addition of indomethacin, a cyclo-oxygenase inhibitor, to patients' MØ culture showed an increase in TNF_α levels. Addition of LTB_4 caused an enhancement in MØ TNF_α responses in some preliminary experiments. These data suggested that the TNF_α enhancing effect of cyclo-oxygenase blockade seen in trauma patients might result from shunting arachidonic acid metabolism through the lipoxygenase pathway and increased LTB_4 levels. As can be seen in Figure 3, the effect of LTB_4 on MØ TNF_α production is independent of any PGE_2 effect. LTB_4 does not enhance all MØ mediator production equally. In those <u>in vivo</u> activated trauma patients with elevated MØ PGE_2 and TNF_α, LTB_4 further enhances TNF_α and IL-6 levels, but has no effect on IL-1 or PGE_2 responses (Figure 4). Normal MØ, which have been $Fc_\gamma R\,I$ crosslinked/activated, also show enhanced IL-6 and TNF_α production when additionally stimulated with LTB_4, but the levels of cytokines produced are still significantly below those seen in the $Fc_\gamma RI$ activated trauma patients' MØ (Figure 4). The final experiment in this series examined the effect of adding both a cyclo-oxygenase and lipoxygenase inhibitor to $Fc_\gamma RI^+$ activated normals' or trauma patients' MØ. Figure 5 illustrates the effect of simultaneous addition of the lipoxygenase inhibitor - nordihydroguairetic acid (NDGA) together with indomethacin to these cells in the presence of MDP. NDGA completely abrogated the enhancing effect of indomethacin. These data indicate that the enhancing effect of indomethacin addition on MØ TNF_α production is due to the indomethacin augmentation of the lipoxygenase pathway and not to its inhibition of cyclo-oxygenase production. These data illustrate that prophylactic treatments (such as indomethacin) to reduce immunosuppressive PGE_2 need to be evaluated for their effects on the total patient cytokine profile. In these experiments, indomethacin decreased immunosuppressive levels of PGE_2, but it also shunted arachidonic acid metabolism toward LTB_4 which resulted in augmenting the levels of TNF_α protein.

In another similar set of experiments, we investigated how TGFβ affected production of TNF_α by trauma patients' MØ. We had previously published (10) that patients' MØ produced excessive TGFβ levels. It was also well established that TGFβ downregulated TNF_α production in normal activated MØ. When we added exogenous TGFβ back to patients' and normals' MØ stimulated with a suboptimal dose of interferon gamma (10U IFN_γ) and MDP, we observed contrasting effects. Exogenous TGFβ addition decreased the levels of TNF_α induced in normals' MØ. However, TGFβ increased the levels of TNF_α bioactivity produced by patients' MØ (Table 5). Both cell-associated and secreted TNF_α levels were increased in the patients' MØ. These data imply that the elevated TGFβ produced by trauma patients' MØ actual enhance their subsequent TNF_α production and provide another mechanism for post-trauma elevation of MØ TNF_α production.

The possible pathological effects of normals' MØ TNF_α production is also ameliorated by shedding of TNFR from the MØ surface during TNF_α production. TNF_α production is coordinated with shedding of the MØ TNFR. The two TNFR can complex with TNF_α protein and prevent TNF_α mediated damage and/or activation of other cells.

Stimulation of normals' MØ with IFN_γ + MDP is known to induce both TNF_α protein and TNFR production and to mediate shedding of the increased TNFR off the MØ surface. This shedding of TNFR from the surface of MØ which are producing TNF_α has two important consequences. First, TNF_α producing MØ lose the capacity to be autocrine stimulated by their own TNF_α. Second, the shed soluble receptors bind and inactivate some of the secreted TNF_α protein, thereby diminishing its potential for damaging bystander cells.

In a series of experiments, levels of TNFR on trauma patients' MØ were assessed by their ability to bind fluoresceinated TNF_α. Patients were retrospectively designated immunocompetent (normal mitogen + PGE_2 levels) or immunoaberrant (depressed mitogen with elevated PGE_2 response). The percent of MØ binding TNF_α and the fluorescent intensity of the MØ (i.e. receptor density) was measured by flow cytometry. This method of TNFR monitoring does not distinguish between TNFR of the 80kD type versus TNFR of the 60kD type. However this

method does allow quantification of total TNFR levels and minimize the non-specific binding through the MØ $Fc_\gamma RI$ that is a problem with using specific anti-TNFR 80 or anti-TNFR 60 antibodies. These experiments produced two different results, depending on the stimulation of the MØ, and at first appeared to be contradictory. When unstimulated MØ from immunoaberrant trauma patients were compared with MØ from paired normals, the level of TNFR was significantly depressed (p=.001 Wilcoxon). Immunocompetent trauma patients were found to express TNFR levels equivalent to normals (Table 6). However, when the patients' and normals' MØ were stimulated with 10U IFN_γ + MDP, the normals' and immunocompetent patients' MØ down-regulated their TNFR levels simultaneous to their production of TNF_α protein (Fig.7). In contrast, the immunoaberrant trauma patients upregulated their TNFR expression (Fig.8). We interpret these data as indicating first that the immunoaberrant patients are initially expressing normal or high levels of TNFR which are masked from detection by already having bound TNF_α protein. Such surface TNF_α - TNFR complexes have been previously described. The normals' and immunocompetent patients' MØ are expressing their uncomplexed TNFR. When the patients' and normals' MØ are stimulated to start producing TNF_α protein, both patient groups and the normals shed their TNFR. However, the immunoaberrant patients are also stimulated to express additional TNFR which are not masked by TNF_α protein and which are not shed. An alternative explanation is that TNFR are being continually shed by the immunoaberrant MØ because of the high level of <u>in vivo</u> activation. When additional stimulation (10 γ + MDP) is given to these highly activated cells, they produce such increased numbers of TNFR that they exceed their ability to be shed. Whatever the mechanism for these results, the data imply that immunoaberrant trauma patients' MØ are expressing high levels of TNFR on their surface while simultaneously secreting TNF_α protein, producing a system for autocrine stimulation and continued TNF_α and other cytokine induction. Such an auto-stimulatory loop would ensure continued high cytokine production by these patients' MØ and could be a mechanism causing the continued high levels of TNF_α observed in SIRS.

The continued high level of TNF_α produced by trauma patients' MØ appears to have multiple causes. Autocrine stimulation through expressed TNFR, loss of PGE_2 sensitivity and increased TNF mRNA stability have all been examined in our laboratory. In addition, MØ failure to respond to regulatory cytokine regulators could allow prolonged MØ production of inflammatory mediators leading to SIRS. Both IL-4 and IL-10 are cytokines which can downregulate a number of inflammatory cytokines and should act to decrease post-trauma MØ activity. We had previously shown that IL-4 can downregulate the MØ TNF_α and PGE_2 levels of immunoaberrant trauma patients (14,15). MØ production of TGF_β is greatly increased post-injury and we have demonstrated that TGF_β acts on patients' MØ to increase their TNF_α levels. Consequently, we examined the ability of exogenous addition of IL-4 to downregulate TGF_β levels in both trauma patients' MØ and normals'. As can be seen in Table 7, IL-4 depressed MØ TGF_β production both in the patients and in the normals. In examining the mechanism for IL-4 downregulation of MØ TGF_β, we showed that TGF_β mRNA was decreased by IL-4 and that this decrease required protein synthesis (Fig.9). These data suggest that IL-4 might be a widely applicable therapy in trauma patients identified as having deregulated cytokine production. The data also imply that patients' own IL-4 levels are either depressed or inadequate for cytokine regulation. We are investigating these possibilities. As an additional possibility, we examined the effect of IFN_γ on TGF_β production by trauma patients' MØ. IFN_γ therapy has been proposed as improving MØ APC function in trauma patients. As can be seen in Table 8, IFN_γ would decrease MØ production of TGF_β, giving another possible explanation for the immuno-enhancing effects reported for IFN_γ therapy.

IFN_γ levels have been reported as depressed after trauma and IFN_γ therapy has appeared beneficial in some trauma models. Most IFN_γ measurements have been made on the patients' peripheral blood mononuclear population, rather than an isolated T cell preparations. Consequently, there is a question of whether T cell IFN_γ production is depressed in this population because of T cell dysfunction, because of MØ inhibitory cytokine depression of T cell IFN_γ induction, or because of lack of appropriate MØ signals to the T cell. The direct question of

T cell dysfunction is examined in a later set of experiments, but we also focused on how MØ induction signals might be altered post-injury, resulting in failure to induce IFN_γ. A recently described monokine, IL-12, has been identified as critical in maximal T cell production of IFN_γ. Consequently, a post-trauma decrease in MØ IL-12 production might result in a failure of T cells to be maximally stimulated to produce IFN_γ. In preliminary experiments using an IL-12 ELISA, there does indeed appear to be decreased MØ IL-12 production in immunoaberrant trauma patients. IL-12 addition has been suggested as a therapeutic treatment to increase T cell activity in HIV infected patients. As a prelude to our investigations into the effect of IL-12 on trauma patients' T cell function, we wished to define how IL-12 addition increased T cell production of IFN_γ. Although it has been reported that the IL-12 induced increase of IFN_γ production by T cells is due to an increase in IFN_γ transcription, these data have been difficult to confirm. In order to more clearly delineate the nature of IL-12 induced augmentation of IFN_γ production, purified human T cells were stimulated with immobilized anti-CD3 and anti-CD4 (dual Ab) in the presence or absence of IL-12 and were monitored for IFN_γ specific mRNA levels and IFN_γ protein.

IL-12 was found to augment IFN_γ production. However, there appeared to be no corresponding increase in IFN_γ mRNA transcription. As little as three hours of induction of T cells with dual Ab plus IL-12 resulted in significant augmentation of IFN_γ protein production as compared to T cells treated with Ab alone. The augmentation of IFN_γ protein production by IL-12 was still apparent at 24 hours post-stimulation. In order to determine if increased IFN_γ protein production was due to an increase in the amount of IFN_γ specific mRNA transcribed by the cells, IFN_γ mRNA levels of the treated (dual Ab $\pm$ IL-12) populations were determined at several time points (Fig.11). IFN_γ mRNA transcription induced by the dual Ab treatment of MØ is apparently augmented by IL-12 during the first two hours of culture. Subsequently, however, measurable mRNA levels in these cells were decreased as compared to the levels of IFN_γ mRNA in the cells treated with dual Ab alone. It is doubtful, therefore, that the prolonged IL-12 induced augmentation of IFN_γ protein production is due only to its effect in increasing transcription. Examination of IFN_γ specific mRNA and IFN_γ protein levels (Figs.12&13), stimulated by dual

Ab ± IL-12, reveals a peak in mRNA production followed by a decrease in detectable mRNA in both the dual Ab and dual Ab/IL-12 stimulated cells. This mRNA peak occurs earlier but is of lesser magnitude in the dual Ab/IL-12 stimulated cells, again indicating that transcriptional augmentation of IFN_γ by IL-12 cannot be the mechanism for increased IFN_γ protein. IFN_γ protein production increases steadily in both populations, but reaches greater concentrations in the IL-12-treated populations. Taken together, these data suggest that addition of IL-12 to a population of anti-CD3/anti-CD4 stimulated T cells induced the cells to rapidly transcribe greater levels of IFN_γ-specific RNA. Additionally, IL-12 apparently induces more rapid and/or efficient translation of this RNA as is demonstrated by the fact that the degree of increased protein production in these cells is not easily attributed solely to the minimal and temporary increase in available RNA. Thus, the IL-12-induced increase in IFN_γ production is likely due to bimodal mechanisms acting at both the transcriptional and translational levels.

We will be evaluating the effect of IL-12 addition to trauma patients' T cells at both the molecular and biological levels to determine if IL-12 can ameliorate T cell deficits in trauma patients. The decreased T cell proliferation to mitogen seen in trauma patients' whole PBMC population could represent a deficit in MØ antigen-presenting or IL-12 function, excessive MØ production of suppressive mediators such as PGE_2 and TGF_β, T cell depletion, or an actual T cell dysfunction. To determine where the defect lies, we have assessed the patients' T cell proliferation in the presence (with the PBMC population) and absence (with the MØ-depleted, SRBC rosette-purified T cell population) of antigen presenting cells (APC). As can be seen in Figure 14 and Figure 15, patients with depressed PHA responses could have either normal/elevated levels of T lymphocyte proliferation (Fig.14), or severely depressed levels of T cell proliferation (Fig.15) in response to anti-CD3 plus anti-CD4, which is our APC-free system. In fact, one patient could exhibit both a normal level of T cell proliferation and a depressed level of T cell proliferation at different post-injury days (Fig.16). These data imply that although MØ dysfunction may be solely mediating some post-trauma depression of induction of T cell proliferation, a true T cell defect may also be present in some severely injured patients. As described above, IL-12 production by MØ or

B lymphocytes increases the proliferation and differentiation of Th1 type $CD4^+$ T lymphocytes and induces IFN_γ and IL-2 secretion. IL-12 was exogenously added in some PHA experiments to determine if it could restore the PHA responses in trauma patients. There was partial restoration of the depressed PHA responses in those patients whose APC-independent proliferation was normal (Fig.17). In contrast, trauma patients having massively depressed T cell proliferation also in our APC-free system showed no increase in PHA responses with in vitro IL-12 addition (Fig.18). Partial restoration of depressed PHA responses by IL-12 in the face of competent T cell function may suggest some depression in MØ IL-12 production in trauma patients as we have already discussed. Additionally, as already discussed, increased post-trauma MØ production of PGE_2 and TGF_β can also inhibit T cell proliferation. Thus, PGE_2 and TGF_β can also be responsible for depressed PHA responses in the face of competent T cell function. This inhibition may be overcome by IL-12 addition and increased T cell activation. The true T cell defect (observed in our APC-free system) might also result from increased T cell production of inhibitors such as TGF_β or IL-10. Alternatively, since activation-induced T cell anergy has been reported after thermal injury (16), the dysfunctional patient's T cells may be anergic due to continued stimulation in the absence of appropriate MØ signals. We are in the process of examining each of these causal mechanisms for T lymphocyte depression.

One of the primary inhibitory regulators produced by T lymphocytes is Interleukin 10. IL-10 can depress both proliferation and activation in T lymphocytes of the Th1 and Th2 types but its primary inhibitory effect is on MØ and Th1 lymphocytes. IL-10 positive effects include stimulation of B cell differentiation and antibody production, as well as protection of activated T lymphocytes from undergoing apoptosis. Since T lymphocytes, MØ, and some B cells can all produce IL-10, and these cell types are differentially affected by trauma, post-trauma IL-10 levels could be increased or decreased depending on time post-injury, clinical course, and cell type examined. There has been some controversy over the role of IL-10 post-injury. IL-10 has been reported as increased in murine splenocytes after hemorrhage, suggesting a negative role for IL-10 (i.e. increasing immunosuppression). However, IL-10 deficient mice are much more susceptible

to septic shock and have greatly elevated TNF_{α} and IL-1 production, suggesting IL-10 is beneficial in controlling SIRS. In contrast, a study of septic (but not trauma) patients reported increased serum IL-10. In our experiments, we investigated the levels of IL-10 in patients' mitogen stimulated PBMC, in MDP stimulated isolated MØ, and in anti-CD3 and anti-CD4 stimulated isolated T lymphocytes. The results appear to reconcile many of the contradictory reports on IL-10 levels after injury.

First, as can be seen in Table 9, trauma patients with depressed mitogen PBMC responses also had lower IL-10 levels than normals after mitogen stimulation. Since MØ are the main producers of IL-10 in stimulated PBMC, we assessed the IL-10 production of isolated patients' versus normals' MØ in the mitogen depressed patients. Here again in the mitogen depressed patients, MØ IL-10 levels were depressed (Table 10). Since most trauma patients' PBMC population have reduced T lymphocyte numbers, it was possible that the reduced IL-10 levels detected were not accurately reflecting the T cell production of IL-10. This is particularly a possibility because we had already shown that patients with depressed PBMC mitogen-induced proliferation could have normal, elevated, or depressed T cell proliferation in the anti-CD3/anti-CD4 system. Since some investigators had seen elevated IL-10 production in murine T cells after hemorrhage but not in the splenocytes, we examined IL-10 production in our patients' isolated T cells stimulated with anti-CD3/anti-CD4. Here the results were surprising. In mitogen depressed patients with normal or elevated T cell proliferation, IL-10 levels were elevated (Table 11). In patients with elevated mitogen responses, the IL-10 levels were even more elevated (Table 12). These data demonstrate that elevated T cell IL-10 levels do occur post-injury, but only in patients who have no T lymphocyte deficiency. These patients appear to have activated T lymphocytes and their post-trauma increased IL-10 may be a normal and beneficial response to control these activated T cells. Since these patients had normal or elevated T cell proliferation, the elevated IL-10 levels could not be responsible for the patients' depressed T cell proliferation in response to mitogens.

In striking contrast, patients with depressed mitogen responses who had depressed T lymphocyte proliferation had a major depression in their T cell IL-10 levels (Table 13). Depression of T cell IL-10 was associated with depressed T lymphocyte function, again arguing that post-trauma elevation in IL-10 is not associated with immunosuppression but with hyper-T cell activation. Of the five patients with major T cell IL-10 depression, four succumbed to SIRS and one had a complicated clinical course and was clinically described as being in "septic shock". The negative effects of depressed IL-10 production in post-trauma MØ is illustrated in Table 14. All trauma patients whose MØ IL-10 levels were depressed also showed major increases in TNF_α production (Table 14).

CONCLUSIONS:

Combining these data, we have developed the following hypotheses. Severe trauma initially activated both T lymphocytes and MØ. This increased activation of T cells in MØ is initially compensated by increasing IL-10 production by T cells and normal IL-10 production by MØ. As MØ continue to produce immunosuppressive substances such as PGE_2 and TGF_β, and lose their production of IL-12, severe T cell dysfunction occurs. This T cell dysfunction may be a result of a combination of effects. First, a lack of sufficient IL-10 levels may fail to protect chronically stimulated T cells from apoptosis, resulting in T cell depletion; second, failure of MØ to properly activate T cells; third, overproduction of MØ derived inhibitors such as PGE_2 and TGF_β which depress T cell activation. The result of this T cell dysfunction is a continued failure to control MØ production of inflammatory cytokines. MØ continually activated by trauma-generated mediators in the absence of IFN_γ, IL-2, IL-4, and IL-10 develop aberrant responses that are no longer sensitive to MØ-derived downregulator signals, such as PGE_2 and TGF_β, and are hyper-responsive to subsequent induction by mediators, such as LTB_4, TNF_α, IL-1, IL-6, etc., which normally are either only mildly stimulated or non-stimulated without other signals. As MØ aberrant activities increase, the MØ undergo receptor changes and activation changes which result in prolongation of inflammatory mediator production (i.e. TNF_α) by changing the mRNA stability. These aberrant MØ have altered receptor expression (i.e. more TNFR, more $Fc_\gamma RI$) which allow

them to be hyperactivated both by autocrine stimulation of the MØ (TNFR) and their increased activation potential ($Fc_{\gamma}RI$). In addition, other MØ markers may be indicative of increased activation potential - LTB_4 receptors may be increased while PGE_2 receptors are decreased.

We are investigating the differential expression of MØ and T cell markers post-injury as a means of rapidly identifying patients with functional aberrations. We are examining altered cytokine and mediator functions of both MØ and T cells as a means of determining what combination of therapy might be appropriate for reversing post-trauma deficiency. IL-10, IL-4, cyclo-oxygenase-lipoxygenase inhibitors, IFN_{γ}, IL-12 and other immune mediators are being evaluated for their effects on a wide cytokine profile, making it less likely that a negative effect would be missed. We will be characterizing cytokine alterations at the protein and molecular level.

In summary, this laboratory is exploring the mechanisms and extent of post-trauma immunopathology at both the biological and molecular levels. Furthermore, we also have a primary commitment to the development of new assay systems and the derivation of possible prophylactic treatments that could be utilized in situations where immediate combat casualty evacuation is impossible and infection, therefore, more likely. Early detection and individually tailored treatment of combat casualties would not only result in a higher survival rate, but also a quicker return to duty. We are committed to translating the data we obtain on aberrant post-trauma MØ and T cell pathways into clinically relevant applications.

Figure 1

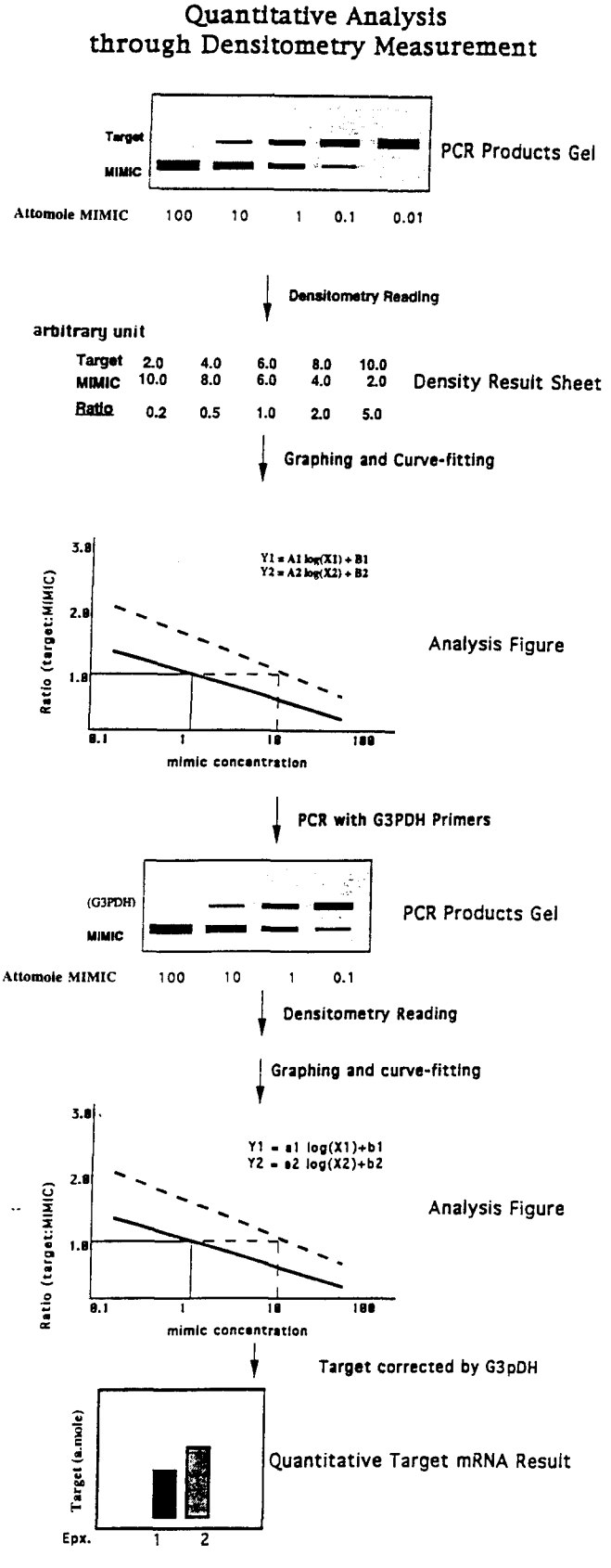
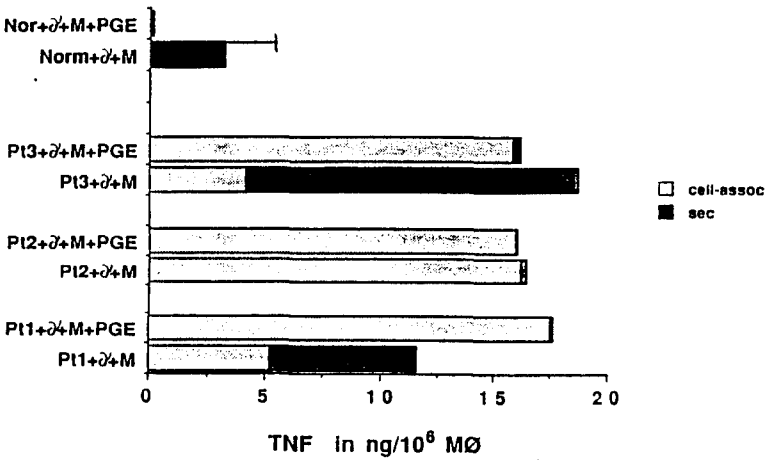

Quantitative Analysis
through Densitometry Measurement
Target
MIMIC
PCR Products Gel
Attomole MIMIC 100 10 1 0.1 0.01
Densitometry Reading
arbitrary unit
Target 2.0 4.0 6.0 8.0 10.0
MIMIC 10.0 8.0 6.0 4.0 2.0
Ratio 0.2 0.5 1.0 2.0 5.0
Density Result Sheet
Graphing and Curve-fitting
Y1 = A1 log(X1) + B1
Y2 = A2 log(X2) + B2
Ratio (target:MIMIC)
mimic concentration
Analysis Figure
PCR with G3PDH Primers
(G3PDH)
MIMIC
PCR Products Gel
Attomole MIMIC 100 10 1 0.1
Densitometry Reading
Graphing and curve-fitting
Y1 = a1 log(X1)+b1
Y2 = a2 log(X2)+b2
Analysis Figure
Target corrected by G3pDH
Target (a.mole)
Epx. 1 2
Quantitative Target mRNA Result

Figure 2

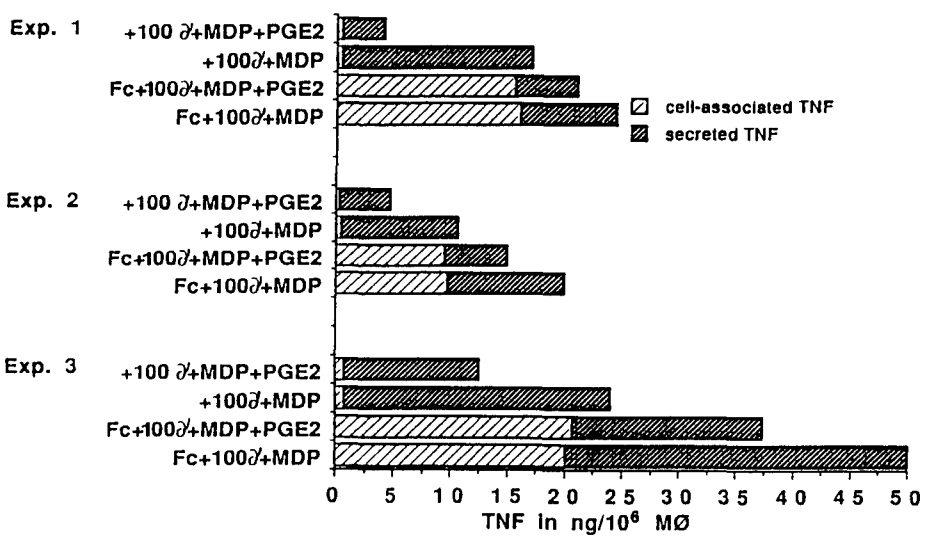
PGE2 Addition Downregulates Both Patients' and Normals' Secreted TNF



10^6/ml trauma patient or normal MØ cultured with 10U IFN_γ plus 20μg/ml MDP in the presence or absence of 10^{-7}M exogenously added PGE2. TNF_α measured in LM bioassay as described. Cell-associated TNF_α (cell-assoc) measured in MØ membrane sonicates while secreted TNF_α (sec) measured in MØ supernates. Normal TNF_α values represent the medium and range for paired normals.

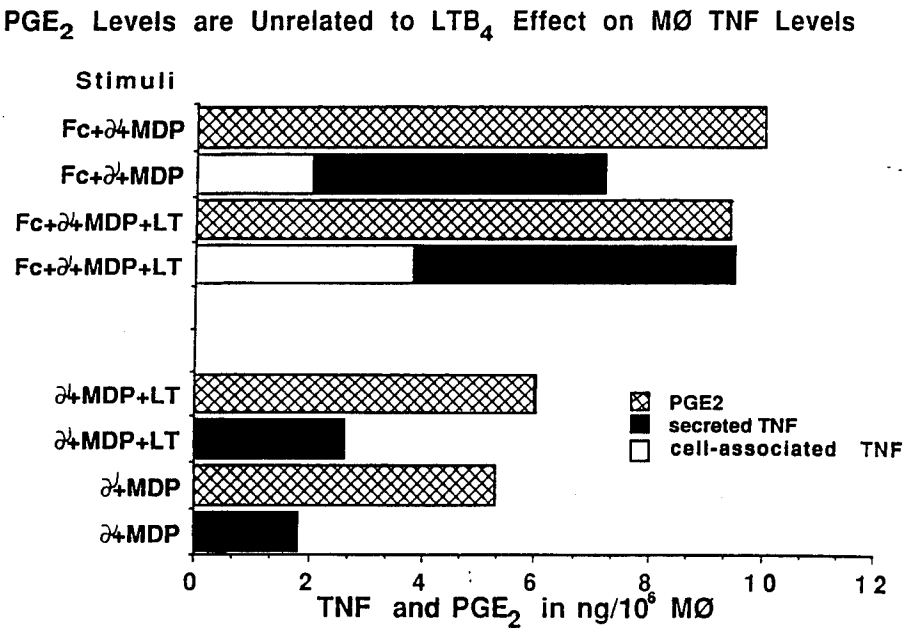
Figure 3

Cell-associated TNF induced by $Fc_\gamma R1$ crosslinking MØ is resistant to PGE2 inhibition while secreted TNF is sensitive



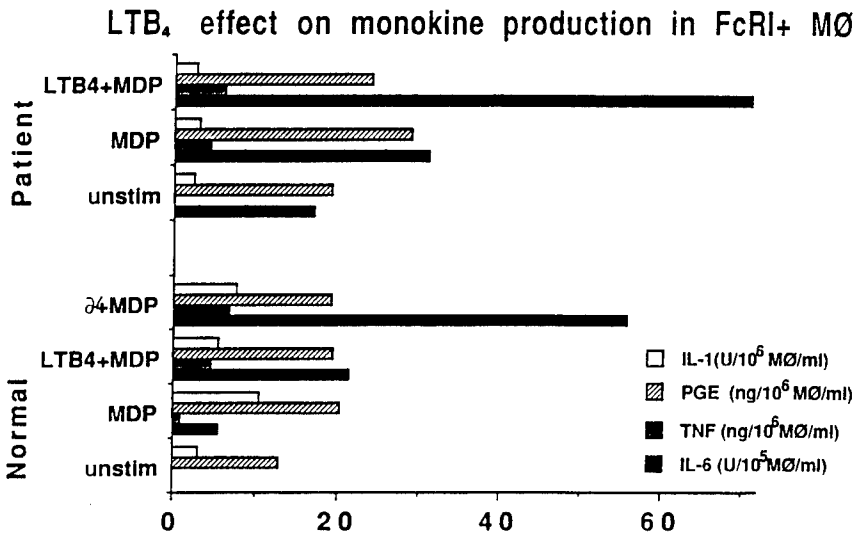
Normal MØ were either stimulated by crosslinking the 72KD IgG receptor during rosetting with anti-Rh coated erythrocytes (Fc) before induction in culture or cultured directly. During culture, both $Fc_\gamma RI$ crosslinking and paired unstimulated MØ were further induced with 100U/ml of IFN_γ (100γ) plus 20μg/ml MDP in the presence or absence of 10^{-7} M PGE_2 cell-associated and secreted TNF_α was assayed as above.

Figure 4



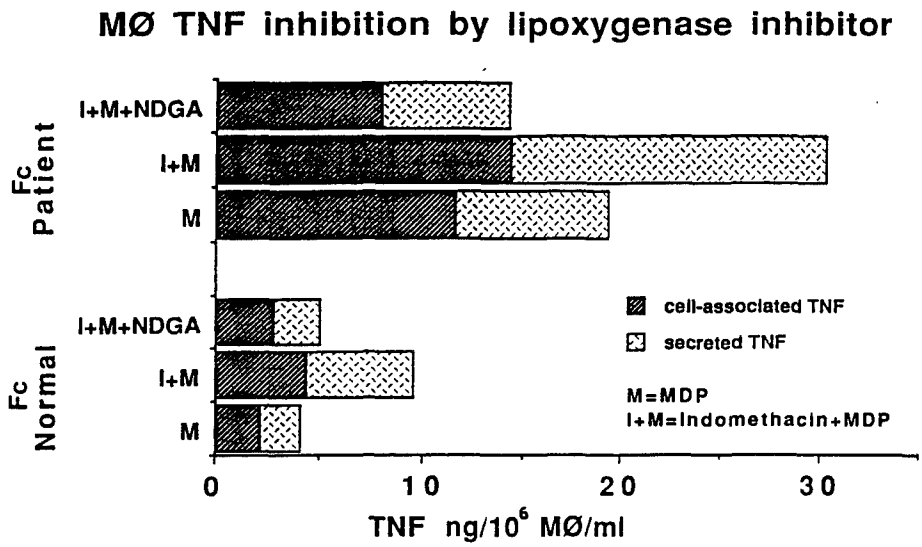
LTB4 stimulates increased cell-associated and secreted TNFα levels in FcγRI cross-linked normal MØ independently of PGE_2 levels. Equal numbers of MØ either FcRI cross-linked (anti-Rh erythrocyte rosetting) or whole MØ population (nonrosetted) were cultured with IFN_γ (100 U/ml) plus MDP (20μg/ml) or IFN_γ (10U/ml) plus MDP (20μg/ml) plus LTB_4 (LT; 10^{-7} M). MØ supernates were assayed for PGE_2 using the ELISA and for secreted TNFα in the LM bioassay. Cell lysates were also assayed for cell-associated TNFα. Both TNFα and PGE_2 are reported in nanograms per 10^6 recovered MØ per milliliter.

Figure 5



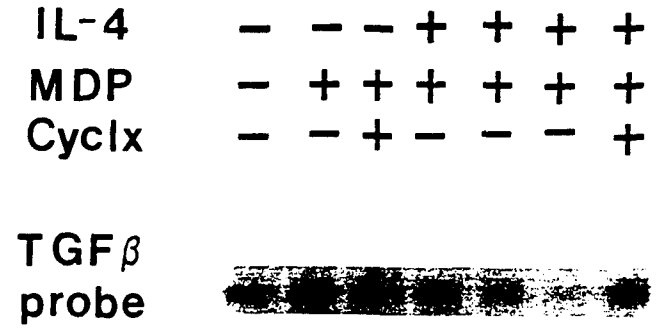
LTB4 effect on monokine production in $Fc_\gamma RI^+$ MØ. $Fc_\gamma RI^+$ MØ were selected by rosetting with anti-Rh coated erythrocytes. Equal numbers of $Fc_\gamma RI^+$ MØ from either patients or normals were cultured for 16 h unstimulated (media only), with MDP (20μg/ml) or, with LTB_4 (10^{-7} M) plus MDP (20μg/ml). Il-1 was measured in the MØ supernates using the D10.G4.1 cell bioassay and reported as units per 10^6 recovered MØ per milliliter. PGE_2 was assayed in the supernates by ELISA and reported as nanograms per 10^6 recovered MØ per milliliter. TNFα was measured using the LM bioassay and presented as nanograms per 10^6 recovered MØ per milliliter. The MØ supernates were also assayed for IL-6 using the B9 cell bioassay and reported as units per 10^5 recovered MØ per milliliter.

Figure 6



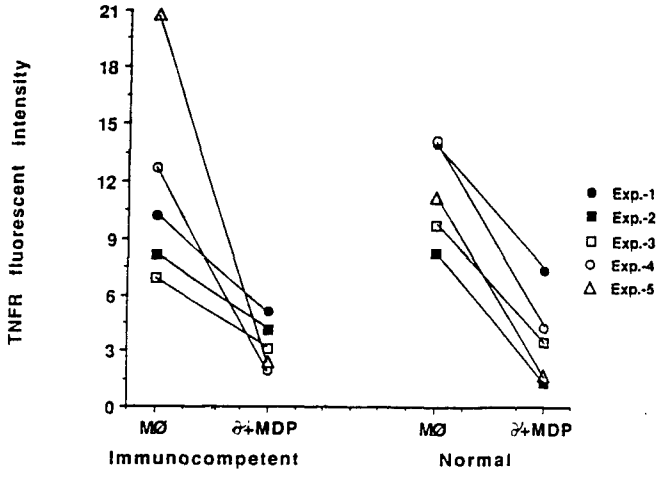
Lipoxygenase inhibitor decreases indomethacin enhanced TNF_α levels. Both normal and patient MØ were FcRI cross-linked (anti-Rh erythrocyte rosetted) and cultured with either MDP alone (M; 20μg/ml) indomethacin (10^{-6} M) plus MDP (I + M; 20μg/ml), or indomethacin (I; 10^{-6} M) plus MDP (20μg/ml) plus the lipoxygenase inhibitor nordihydroguaiaretic acid (I + M + NDGA; 40μM). After 16 h of culture, both secreted and cell-associated TNF_α were measured using the LM cell bioassay. TNF_α is presented as nanograms per 10^6 recovered MØ per milliliter.

Figure 9



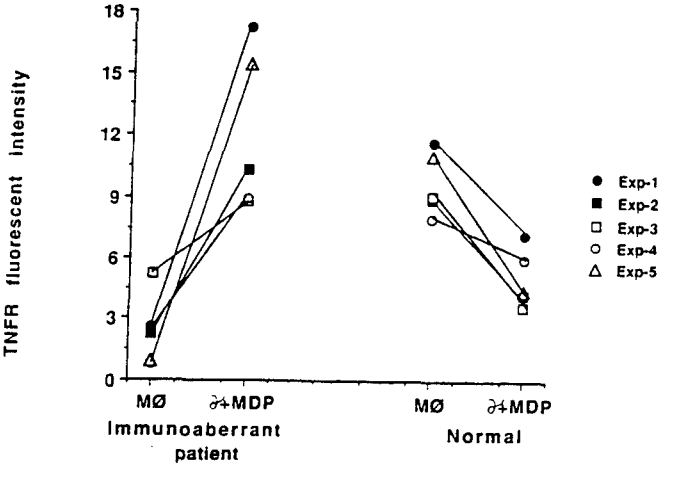
IL-4 downregulation of MØ $TGF_{\beta 1}$ is cycloheximide sensitive. A Northern blot of total MØ RNA was probed with ^{32}P $TGF_{\beta 1}$ or β-actin cDNA. Human peripheral blood MØ were cultured with medium alone (lane 1), with 20 μg/ml MDP (lane 2), with MDP and 1 μg/ml cycloheximide (lane 3) and MDP plus 2.5 ng/ml IL-4 (lane 4), with MDP plus 5.0 ng/ml IL-4 (lane 5), with MDP plus 10 ng/ml IL-4 (lane 6), or with MDP plus 10 ng/ml IL-4 plus 1 μg/ml cycloheximide (Lane 7).

Figure 7



Stimulation decreases TNFR expression on immunocompetent patients' MØ. Peripheral blood monocytes from normals and immunocompetent patients were analyzed both unstimulated (MØ) and after 18 h stimulation with 10 U/ml IFNγ plus 20 μg/ml MDP (γ+*MDP*). Data represent five out of ten paired normal and immunocompetent patient samples. TNFR was measured using phycoerythrin-labeled TNFα gating on forward scatter versus MY4 (CD14) positivity and reported as fluorescent intensity. No significant difference was found between immunocompetent patients (n = 10) and paired normals.

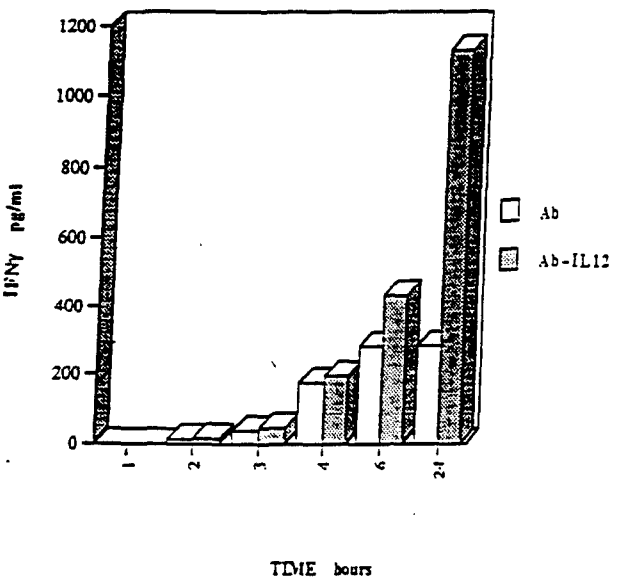
Figure 8



Stimulation increases TNFR expression in immunoaberrant patients' MØ but not normals' MØ. Peripheral blood monocytes from 13 paired normals and immunoaberrant patients (depressed mitogen response and elevated PGE_2) were analyzed both unstimulated and after 18 h stimulation with 10 U/ml IFNγ plus 20 μg/ml MDP. TNFR was measured using phycoerythrin-labeled TNFα gating on forward scatter versus MY4 (CD14) positivity and reported as fluorescence intensity. Data shown from five representative experiments out of 13. *p* value for control unstimulated to immunoaberrant unstimulated n = 13 < .001, control unstimulated to control IFNγ + MDP < .001, unstimulated immunoaberrant patient to paired IFNγ + MDP immunoaberrant patient <.003, and control IFNγ + MDP to immunoaberrant patient IFNγ + MDP <.001. *p* values calculated for 13 experiments.

Figure 10

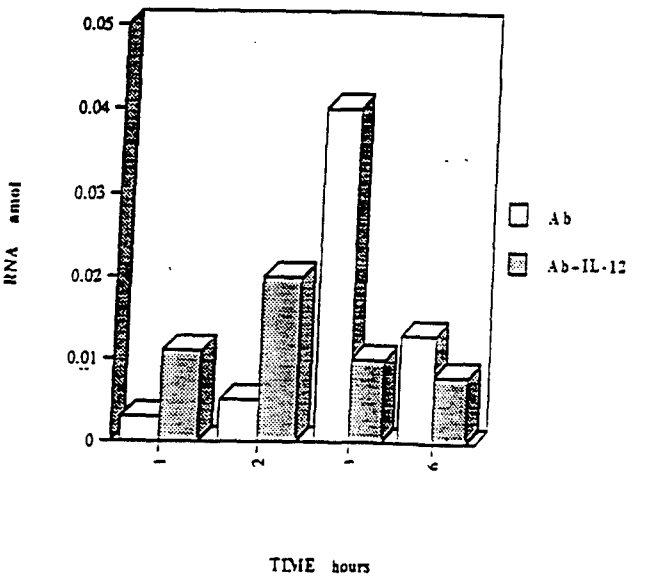
Production of IFN-γ by Cells Stimulated by Anti-CD3 and Anti-CD4 With and Without Addition of IL-12.



Cells were incubated in wells coated with anti-CD3 and anti CD4 either without (white) or with (shaded) the addition of IL-12 to a final concentration of 100 U/ml. IL-12. At indicated times, supernatants were removed and analyzed in an IFN-γ ELISA to determine levels of IFN-γ produced.

Figure 11

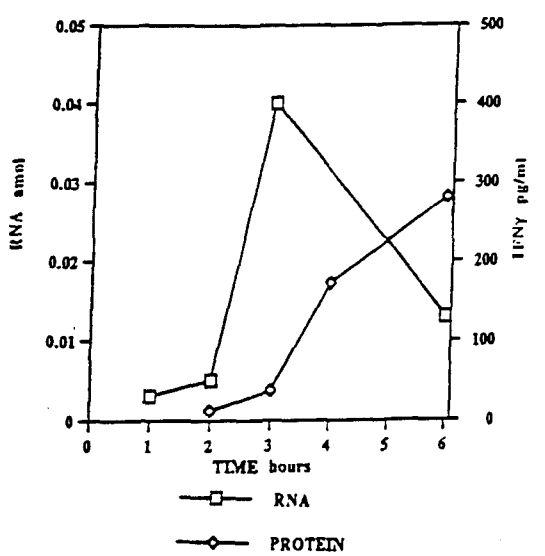
IFN-γ Specific RNA Levels in Cells Stimulated by Anti-CD3/Anti-CD4 With and Without Addition of IL-12.



Cells were incubated in wells coated with anti-CD3 and anti-CD4 either without (white) or with (shaded) the addition of IL-12 to a final concentration of 100 U/ml. At indicated times, cells were lysed and whole cell RNA was reverse transcribed and used in competitive PCR to determine IFN-γ specific RNA levels.

Figure 12

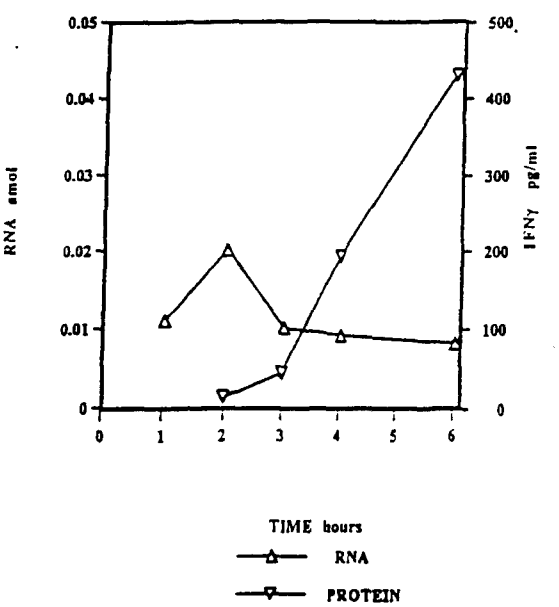
IFN-γ Specific RNA Levels and Production of IFN-γ by Cells Stimulated with Anti-CD3 and Anti-CD4.



Cells were incubated in wells coated with anti-CD3 and anti-CD4. At indicated times, supernatants were removed and analyzed in an IFN-γ ELISA to determine levels of IFN-γ produced(PROTEIN). Cells were lysed and whole cell RNA was reverse transcribed and used in competitive PCR to determine IFN-γ specific RNA levels(RNA).

Figure 13

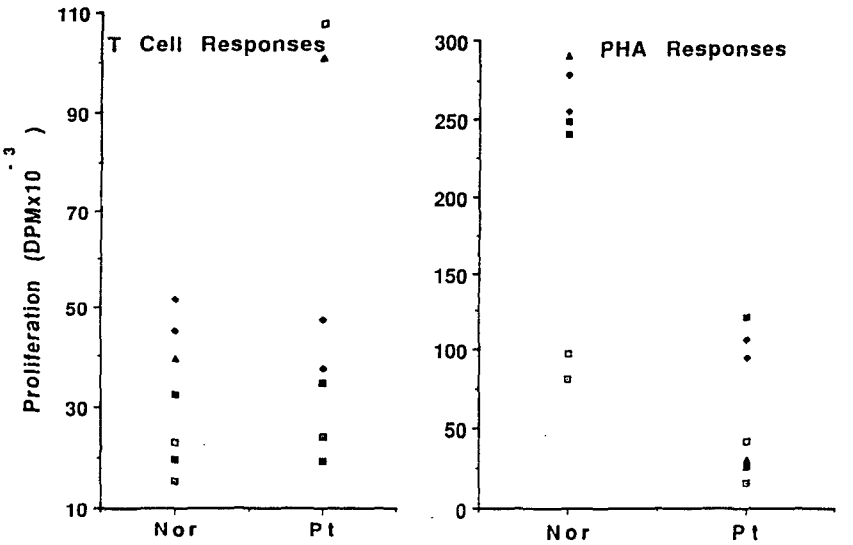
IFN-γ Specific RNA levels and Production of IFN-γ by Cells Stimulated With Anti-CD3/Anti-CD4 and IL-12.



Cells were incubated in wells coated with anti-CD3 and anti-CD4 with 100 U/ml IL-12. At indicated times, supernatants were removed and analyzed in an IFN-γ ELISA to determine levels of IFN-γ produced (PROTEIN). Cells were lysed and whole cell RNA was reverse transcribed and used in competitive PCR to determine IFN-γ specific RNA levels (RNA).

Figure 14

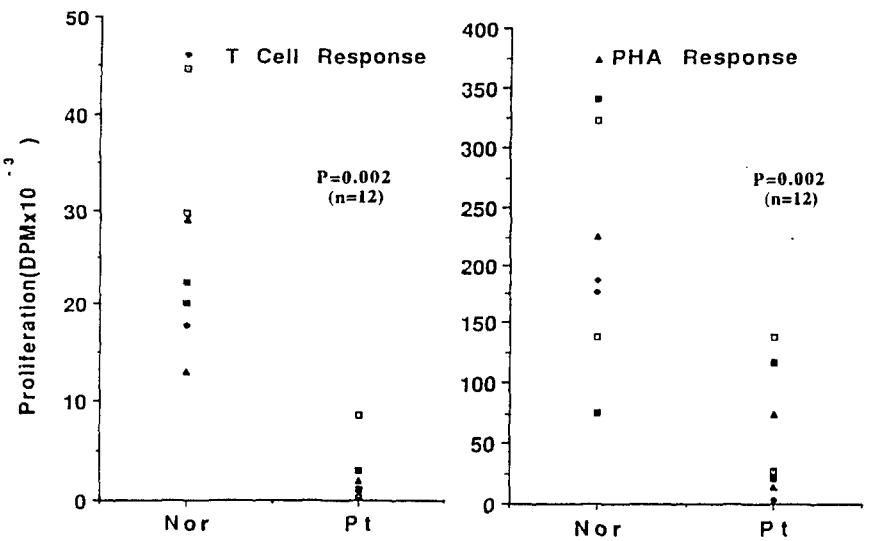
Patients with depressed PHA but normal or elevated T cell responses



Normals' and Patients' PBMC (PHA response) and pure T cells (T cell response) were assessed for proliferation (^{3}H-TdR incorporation) in 72 hours of culture in response to PHA and immobilized anti-CD3 + anti-CD4 respectively and expressed as DPM.

Figure 15

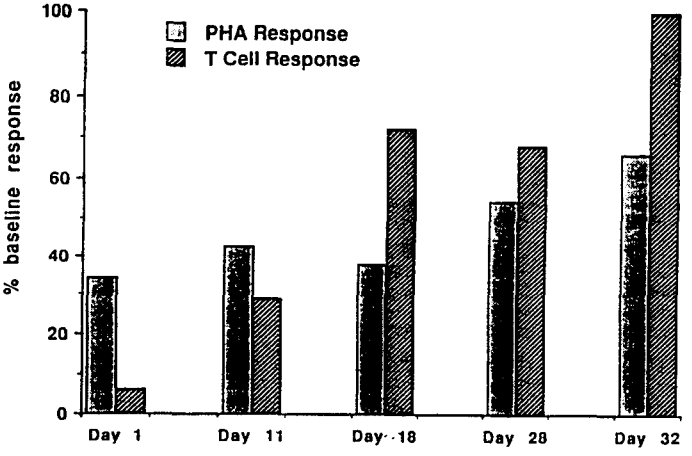
Trauma patients with both depressed PHA and T cell responses



Normals' and Patients' PBMC (PHA response) and pure T cells (T cell response) were assessed for proliferation (^{3}H-TdR incorporation) in 72 hours of culture in response to PHA and immobilized anti-CD3 + anti-CD4 respectively and expressed as DPM.

Figure 16

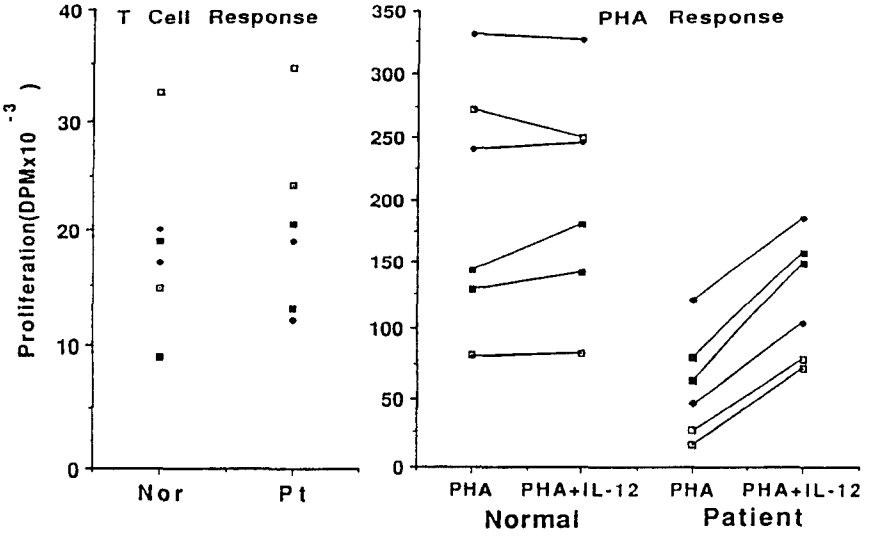
One patient can experience both MØ & T cell dysfunction, only MØ dysfunction and normal proliferation at different post injury days



PHA and T cells responses were assessed in one patient at different post injury days and the % baseline responses calculated as 100 - (A-B) / A x 100, where A = proliferation of paired normal, and B = proliferation of the patient.

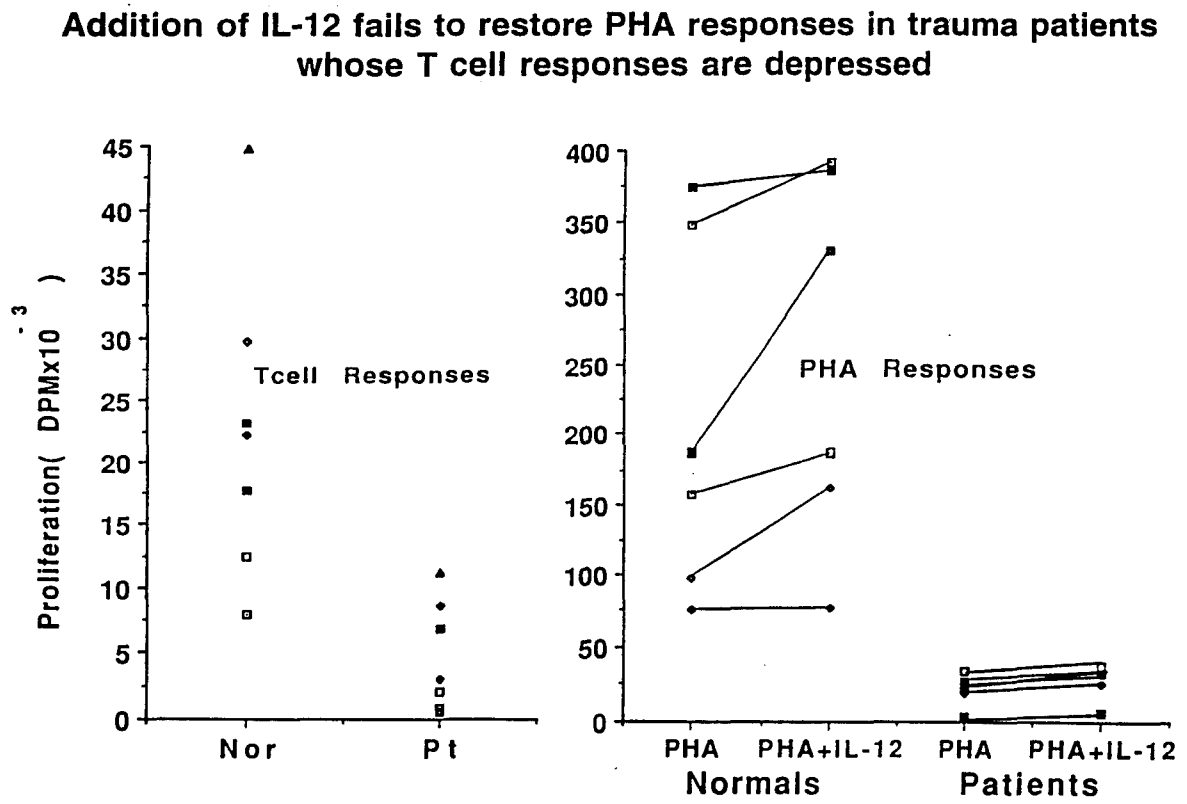
Figure 17

Addition of IL-12 partially restores PHA responses in trauma patients whose T cell responses are intact.



Normals' and Patients' PBMC (PHA response) and pure T cells (T cell response) were assessed for proliferation (^{3}H-TdR incorporation) in 72 hours of culture in response to PHA and immobilized anti-CD3 + anti-CD4 respectively and expressed as DPM. IL-12 was added in PHA experiments at a concentration of 100 U/ml.

Figure 18



Normals' and Patients' PBMC (PHA response) and pure T cells (T cell response) were assessed for proliferation (^{3}H-TdR incorporation) in 72 hours of culture in response to PHA and immobilized anti-CD3 + anti-CD4 respectively and expressed as DPM. IL-12 was added in PHA experiments at a concentration of 100 U/ml.

Table I

Patient	Injury	PGE_2[a]	TNF[b]	PHA[c]
1	Burn	6.9→389.7	5.0	-73%
2	Burn[d]	15.0→ 54.5	3.4	-66%
3	Trauma	142.0	2.1	-45%
4	Trauma	52.8→269.5	2.5	-49%
5	Trauma	4.3	0.9	-46%
6	Trauma	4.6→ 37.0	0.1	-12%
7	Trauma	22.2→ 41.5	0.42	-20%
8	Burn	12.7→ 84.3	22.4	-52%
9	Burn	1.9→128.4	16.9	-86%
10	Trauma[d]	0.12→ 1.4	10.9	-83%
11	Burn	0.9→ 1.4	0.0	+59%
12	Trauma	18.0	0.0	-60%
13	Burn[d]	19.5	13.4	+81%
14	Trauma	1.3→139	87.4	–20%
15	Trauma	3.6	0.0	- -
16	Trauma	1.9→ 18.8	59.3	-83%
17	Trauma	1.2→111.7	0.74	-69%
18	Burn	63.0	27.4	-74%
19	Trauma	1.4	0.0	- -
20	Trauma	31.6→77.3	18.1	-66%
21	Burn	2.7→53.8	1.9	-37%
22	Trauma	2.5	1.3	-35%
23	Burn	0.65→ 8.4	0.39	-53%
24	Burn	5.7→70.8	28.8	-81%
25	Trauma[d]	0.16→15.8	15.1	-98%
26	Trauma	2.0→166.4	43.7	-83%
27	Trauma	8.8	24.4	-56%
28	Burn	0.25→ 1.3	2.18	-54%
29	Trauma[d]	0.46→18.6	166.6	-75%
30	Burn	0.08→22.7	11.6	-67%
31	Burn	0.72	1.2	-45%
32	Trauma	0.16→8.0	0.72	-94%
33	Burn	2.8→16.5	4.96	-75%
34	Burn	51.2	0.0	-45%
35	Burn	0.274→3.5	2.99	-85%*
36	Trauma	3.0	0.0	-1%*
37	Burn	0.1→3.2	2.11	-89%*
38	Trauma	--	--	-82%
39	Burn	0.25→6.3	8.79	-96%*
40	Trauma[d]	--	--	-65%
41	Burn	--	--	-13%
42	Burn	.07→.62	2.28	-85%*
43	Burn[d]	3.8	31.1	-71.3%
44	Burn	0.53→.94	44.2	-50%*
45	Burn	1.4→35.6	9.4	-28%*
46	Burn	0.98→1.0	1.46	–88%

a. Maximal change in MØ prostaglandin E_2 (PGE_2) levels post-injury. PGE_2 is measured in a sensitive ELISA assay and expressed as ng/10^6 MØ/ml.

b. Maximal levels of tumor necrosis factor (TNF) (secreted plus cell-associated) production by unstimulated patients' MØ. TNF is measured in the L-M cell bioassay and expressed as ng/10^6ml.

c. Maximal percent change of the patient mononuclear cells proliferation response to 2µg/ml phytohemagglutinin mitogen compared to the normals.
* compared over time to baseline value.

d. This patient expired.

Table 2 - Patients with increased MØ TNFα bioactivity [a] also have increased MØ TNFα mRNA levels [b]

TNFα mRNA levels in attomoles/10^6 MØ				TNFα bioactivity in ng/10^6 MØ			
Unstim [c]		+MDP [d]		Unstim [e]		+MDP [f]	
Norm	Pt	Norm	Pt	Norm	Pt	Norm	Pt
0.24	0.51	0.51	0.91	U.D. [g]	8.79	U.D.	16.55
0.62	2.88	---		U.D.	14.36	---	
0.22	0.61	0.38	4.61	U.D	14.40	U.D.	26.40
0.21	0.47	0.70	2.38	U.D.	1.20	U.D.	3.90
0.35	0.66	0.83	3.96	U.D.	5.60	U.D.	8.80
0.73	1.34	1.02	2.93	U.D.	2.30	2.5	6.50
0.90	3.64	1.32	7.84	U.D.	4.70	1.4	36.30
0.21	0.64	0.69	3.89	U.D.	1.30	U.D.	4.80
0.90	2.26	1.32	4.45	U.D.	7.10	U.D.	16.80
0.84	2.27	2.90	10.10	U.D.	7.20	1.3	26.10
P = 0.005 [h]		P = -0.007		P = 0.005		P0.007	

a. 10^6 MØ isolated from either trauma patients (pt) or paired normal (norm) controls were assayed after 18 hrs of culture in the LM bioassay. Total TNFα activity in ng/10^6 MØ was calculated from 6 dilutions of the MØ supernate (secreted) plus 6 dilutions of MØ lysates (cell associated) versus a TNFα standard.

b. mRNA levels in attomoles/10^6 MØ as calculated from the minic PCR as shown under methods. Corrected for mRNA differences using hG_3PDH.

c. PBMC cultured 1 hr then nonadherent cells removed. 3 - 6x10^6 isolated MØ cultured an additonal 2 hrs in RPMI 1640 + 15% FBS on microexudate coated plates.

d. 3 - 6x10^6 MØ cultured 2 hr with 20μg/ml muramyl dipeptide (MDP).

e. MØ isolated after 1.5 hrs of PBMC culture on microexudate coated plates then isolated adherent MØ cultured an additional 16 hrs in RPMI + 15% FBS.

f. MØ isolated as above cultured 16 hrs with MDP 20μg/ml.

g. Undetectable.

h. Probability determined using Wilcoxon non-parametric statistics.

Table 3 - Changes in mRNA levels of trauma patients' MØ over time post-injury doesn't result from transcriptional/translational inhibitor loss

TNFα mRNA levels in attomoles/10^6 MØ [a]

Patient with elevated TNFα bioactivity [b]

Post Injury Day	Unstimulated [c] Norm	Pt	MDP [d] Norm	Pt	MDP + CHX [e] Norm	Pt
3	0.32	0.39	0.43	0.59	0.90	1.56
7	0.42	0.65	0.89	1.62	1.25	7.77
9	0.80	3.64	1.30	7.84	2.21	16.54
13	0.71	1.50	1.05	2.21	2.80	5.35
19	0.55	0.74	0.86	1.14	2.10	4.25
23	0.61	0.95	0.98	1.69	1.94	2.53
33	0.43	0.65	1.10	1.17	- -	- -

Patient with minimally elevated TNFα bioactivity

	Norm	Pt	Norm	Pt	Norm	Pt
1	0.43	0.69	0.92	1.10	- -	- -
11	0.61	0.93	0.98	2.05	1.70	3.05
15	0.14	0.37	0.24	0.56	0.47	0.70
30	0.19	0.15	0.38	0.44	- -	- -

a. Attomoles of TNFα assessed in PCR mimic system as described under methods corrected with G_3PDH.
b. Patients' total TNFα bioactivity in LM bioassay increased more than 2 x paired normal response.
c. Isolated MØ cultured 2 hrs. in medium alone.
d. MØ cultured 2 hrs. with 20μg/ml MDP.
e. Isolated MØ cultured 2 hrs.+ 20μg/ml MDP+1μg/10^6 MØ cycloheximide.

Table 4 - Patients with increased MØ TNFα bioactivity and increased MØ TNFα mRNA can have either increased or unchanged mRNA stability

4 a - Patients with increased mRNA and increased mRNA stability

	Unstimulated [a]		MDP Induced [b]		MDP + CHX [c]	
	Norm	Pt	Norm	Pt	Norm	Pt
mRNA @ 2 hrs. [d]	0.21	0.47	0.69	2.38		
mRNA @ 16-18 hrs. [e]	0.02	0.08	0.01	0.28	----	
% residual [f]	9.5%	17%	2%	12%		
mRNA @ 2 hrs.	0.31	1.04	0.50	1.58	0.71	3.00
mRNA @ 16-18 hrs.	0.03	0.14	0.02	0.19		
% residual	9.7%	13%	4%	12%		
mRNA @ 2 hrs.	0.73	1.34	1.02	3.43	1.29	4.72
mRNA @ 16-18 hrs.	0.04	0.88	0.05	1.67		
% residual	6%	65%	5%	49%		
mRNA @ 2 hrs.	0.42	0.65	0.89	1.62	1.25	7.77
mRNA @ 16-18 hrs.	0.02	0.18	0.02	0.29		
% residual	5%	16%	2%	18%		
mRNA @ 2 hrs.	0.50	0.89	0.71	2.28		
mRNA @ 16-18 hrs.	0.05	0.29	0.03	0.39	----	
% residual	10%	32%	4%	17%		

4 b - Patients with increased mRNA but normal mRNA stability

	Unstimulated		MDP Induced	
Norm Pt	Norm	Pt		
mRNA @ 2 hrs.	0.43	1.01	0.88	1.27
mRNA @ 16-18 hrs.	0.05	0.13	0.04	0.08
% residual	12%	12%	5%	6%
mRNA @ 2 hrs.	0.43	0.98	1.06	2.30
mRNA @ 16-18 hrs.	0.02	0.05	0.01	0.07
% residual	5%	5%	1%	3%
mRNA @ 2 hrs.	0.21	0.638	0.70	3.89
mRNA @ 16-18 hrs.	0.02	0.056	0.008	0.07
% residual	9.5%	9%	1%	2%
mRNA @ 2 hrs.	0.38	0.77	0.84	1.46
mRNA @ 16-18 hrs.	0.04	0.09	0.010	0.05
% residual	11%	12%	1%	3%
mRNA @ 2 hrs.	0.27	0.34	0.37	0.82
mRNA @ 16-18 hrs.	0.03	0.04	0.05	0.11
% residual	11%	12%	13%	13%
mRNA @ 2 hrs.	0.43	0.50	0.69	1.02
mRNA @ 16-18 hrs.	0.05	0.06	0.09	0.12
% residual	12%	12%	13%	12%

a. Isolated patient (pt) or paired normal (norm) MØ cultured for 2-18 hrs. in RPMI 1640 + 15% FBS.
b. Isolated patient or normal MØ cultured in 20μg/ml muramyl dipeptide (MDP).
c. Isolated patient or normal MØ cultured in 1μg/10^6 MØ cycloheximide.
d. MØ TNFα mRNA levels in attomoles/10^6 MØ after 2 hrs. of culture as determined in mimic PCR system corrected for total mRNA differences with G_3PDH.
e. MØ TNFα mRNA levels remaining after 16-18 hrs. of culture in attomoles/10^6 MØ.
f. Percent of 2 hrs. TNFα mRNA still detectable in patient or normal MØ after 16-18 hrs. of culture.

Table 5 – $TGF\beta$ increases MØ $TNF\alpha$ levels in $IFN\gamma$ + MDP stimulated trauma patients' MØ while decreasing $TNF\alpha$ in controls' MØ [a]

	TNF bioactivity secreted/cell-associated in ng/10^6 MØ			
	Nor	Pt	Nor	Pt
10 $IFN\gamma$ + MDP	15.8/1.6	23.1/4.5	4.0/0	10.2/24.7
10 $IFN\gamma$ + MDP + $TGF\beta$	4.9/2.1	32.4/3.5	3.1/0	11.4/27.4
10 $IFN\gamma$ + MDP	9.5/5.7	38.1/7.3	11.4/2.6	8.7/24.3
10 $IFN\gamma$ + MDP + $TGF\beta$	5.9/4.7	38.2/7.8	6.9/3.2	12.2/29.6
10 $IFN\gamma$ + MDP	15.1/0	38.1/10.3	2.1/0	10.5/0.3
10 $IFN\gamma$ + MDP + $TGF\beta$	12.4/0	105.8/16.2	1.6/0	17.1/0.3

a. $TNF\alpha$ activity in LM bioassay of $3x10^6$ normal control (Nor) or trauma patients' (Pt) MØ stimulated by 10U/ml $IFN\gamma$ + 20µg/ml MDP in the presence or absence of 2.4ng/ml $TGF\beta$. Secreted $TNF\alpha$ assayed in MØ supernates and cell-associated $TNF\alpha$ in MØ lysates.

TABLE 6 **Altered expression of MØ TNFR* in immunoaberrant trauma patients‡**

	Median TNFR fluorescent intensity in log fluorescence		
	Control (n = 23)	Immunoaberrant (n = 13)	Immunocompetent (n = 10)
Unstimulated§	10.8 (7.5–17.4)	4.2 (0.8–8.5)	10.4 (20.8–6.9)
10 U IFNγ + MDP¶	3.5 (7.9–1.1)	8.9 (17.2–4.9)	4.6 (8.1–1.2)

MØ TNFR levels increase after IFNγ + MDP stimulation. $p < .001$, control unstimulated to immunoaberrant unstimulated; p = NS, control unstimulated to immunocompetent; $p < .001$, control unstimulated to control IFNγ + MDP; $p < .003$, unstimulated immunoaberrant patient to IFNγ + MDP immunoaberrant patient; $p < .001$, control γ + MDP to immunoaberrant Patient γ + MDP; p = NS, control γ + MDP to immunocompetent patient γ + MDP.

* Mean fluorescent intensity for TNFR as measured using phycoerythrin-tagged TNFα gaiting on forward scatter versus MY4 (CD14) positivity.

‡ Patients designated as immunoaberrant if their mitogen response was depressed >35% from their admission sample or from their discharge samples and their PGE_2 levels were elevated.

§ Unstimulated MØ were freshly isolated and measured for TNFR before adherence.

¶ Adherent MØ stimulated with 10 U/ml IFNγ plus 20 µg/ml MDP for an 18 h period.

Table 7 - IL-4 Downregulation of Trauma Elevated TGF_β [a] Levels in Patients' MØ

	Exp.1	Exp. 2	Exp. 3	Exp. 4
Nor + MDP [b]	8.9	45.6	23.1	15.2
Nor + MDP + IL-4 [c] (25ng)	< 10.0	< 10.0	8.8	< 10.0
Pt + MDP	43.0	85.3	89.7	126.0
Pt + MDP + IL-4 (25ng)	< 10.0	44.8	18.2	47.0

	Exp. 5	Exp. 6	Exp. 7
Nor + MDP	38.5	58.0	42.7
Nor + MDP + IL-4 (5ng)	15.5	22.5	--
Nor + MDP + IL-4 (25ng)	11.2	1.0	6.4
Pt. + MDP	124.6	202.1	63.5
Pt + MDP + IL-4 (5ng)	27.5	118.7	--
Pt + MDP + IL-4 (25ng)	19.5	42.5	17.9

a. TGF_β in pM/10^6 recovered MØ/ml as measured in Mink Lung assay.

b. 10^6 MØ cultured for 16 hours in Iscove's media and muramyl dipeptide.

c. The statistical difference between Nor + MDP and Pt + MDP determined by the Wilcoxon test is P = 0.018; and between Nor + MDP and Nor + MDP & IL-4 (25 ng) is P = 0.018; the P between Pt + MDP and Pt + MDP & IL-4 (25ng) is 0.018.

Table 8 - Excess Production of TGF_β by Patients' MØ [a] is Downregulated by IFN_γ [b]

	Exp. A	Exp. B	Exp. C	Exp. D
Nor MØ	30.7	15.7	108.9	<10.0
Nor MØ + MDP	51.4	119.1	287.4	24.7
Nor MØ + 10 γ + MDP	70.4	145.9	65.8	41.8
Nor MØ + 100 γ + MDP	23.5	81.2	31.8	106.5
Patient MØ	222.2	426.1	2219.0	303.0
Patient MØ + MDP	371.4	1007.1	3353.1	391.0
Patient MØ + 10 γ + MDP	366.4	818.0	330.0	231.8
Patient MØ + 100 γ + MDP	164.0	635.3	206.0	--

a. TGF_β in pM/10^6 recovered MØ from either normals' or trauma patients' MØ.

b. 10^6 MØ cultured for 16 hours with Iscoves Media, alone or either with 20μg/ml MDP, or primed for 2 hours with either 10U/ml or 100U/ml IFN_γ plus MDP, or primed with 100U/ml MDP plus MDP.

Table 9 Depressed IL-10 production by immunosuppressed trauma patients' PBMC

Experiment	% Decrease Proliferation [b]	IL-10 [a] (ng/10^6 PBMC/ml) Normal	Patient [c]
1.	41	5.350	1.160
2.	65	4.005	1.850
3.	81	2.850	0.320
4.	42	3.600	<0.005
5.	96	2.040	0.460
6.	66	4.520	0.380
7.	57	4.080	0.930
8.	98	4.600	<0.005
9.	85	5.100	0.360
10.	71	2.900	1.470
11.	71	4.600	0.790
12.	67	1.640	0.340
		P Value [d] =	0.0022

a. PBMC were cultured in 96 - well plate ($2x10^5$cells/200 µl/well) in presence of PHA. After 24 hours of culture, supernates were harvested and assessed for IL-10 by ELISA.

b. PHA induced proliferation calculated from [^{3}H] thymidine incorporation using the formula A-B/A x 100 where A= counts for normal, B= counts for patient; and the patients having > 30% decrease in proliferation were considered immunosuppressed.

c. 12 samples were collected from 6 patients at different post injury days.

d. Statistical significance (P) between normal and patient values determined by Wilcoxon non-parametric test.

Table 10 Depressed MØ IL-10 production in immunosuppressed trauma patients

		MØ IL-10 [a] (ng/10^6-MØ/ml)			
	% Decrease	Unstimulated [b]		MDP [c]	
Experiment	Proliferation [d]	Normal	Patient [e]	Normal	Patient
1.	39	2.775	0.031	4.687	0.075
2.	91	3.186	< 0.005	4.329	<0.005
3.	73	0.621	0.083	2.451	0.134
4.	67	0.265	< 0.005	1.139	0.033
5.	70	0.325	< 0.005	1.022	<0.005
6.	70	0.942	0.148	2.809	0.230
7.	42	2.081	< 0.005	3.024	0.099
8.	51	0.480	0.013	1.313	0.096
9.	72	0.623	< 0.005	2.053	0.357
10.	65	0.508	0.089	1.934	0.604
11.	80	3.781	< 0.005	10.466	0.461
12.	55	3.559	0.686	6.195	0.700
13.	79	0.860	< 0.005	2.216	0.493
14.	42	3.107	0.149	6.964	0.704
15.	98	2.515	< 0.005	5.944	<0.005
16.	71	2.515	0.268	5.944	0.755
		P Value [f] = 0.0004		P Value = 0.0004	

a. IL-10 levels in the MØ culture supernates measured by ELISA.

b. MØ ($3x10^6$ cells/3ml) cultured in medium alone for 16 hours.

c. MØ ($3x10^6$ cells/3ml) cultured in medium and MDP (20 µg/ml) for 16 hours.

d. PHA induced proliferation calculated from [^{3}H] thymidine incorporation using the formula A-B/A x 100 where A= counts for normal, B= counts for patient; and the patients having >30% decrease in proliferation were considered immunosuppressed.

e. 16 samples were collected from 12 patients at different post injury days.

f. Statistical significance (P) between normal and patient values determined by Wilcoxon non-parametric test.

Table 11 T Cell IL-10 production in trauma patients having normal or elevated T cell proliferation [a]

	T Cell Proliferation (DPMx10^{-3})		IL-10 [b] (pg/10^6 T Cells/ml)	
Experiment	Normal	Patient	Normal	Patient [c]
1	17	13	117	162
2	14	24	34	135
3	9	20	10	20
4	19	12	5	14
5	53	70	62	744
6	45	47	156	468
7	19	19	63	93
8	22	26	46	46
			P Value [d] =	0.018

a. T cell proliferation was assessed in a [^{3}H] thymidine incorporation assay by culture of monocyte-depleted SRBC-rosetted T cells ($2x10^5$cells/200µl/well) in presence of immobilized anti-CD3 (1.5µg/well) plus anti-CD4 (1µg/well) for 72 hours and expressed as DPM.

b. IL-10 levels in the supernates of T cells cultured in presence of immobilized anti-CD3 (1.5µg/well) plus anti-CD4 (1µg/well) for 24 hours, were measured by ELISA.

c. 8 samples were collected from 5 patients at different post injury days.

d. Statistical significance (P) between normal and patient values were determined by Wilcoxon non-parametric test.

Table 12 Increased mitogen induced and T cell proliferation concomitant to increased IL-10

Exp. No.	% Increase PHA-response [a]	T cell proliferation (DPM x 10^{-3}) [b]		IL-10 (pg/10^6T cells/ml) [c]	
		Normal	Patient [d]	Normal	Patient
1	485	49	104	122	175
2	100	17	47	8	94
3	89	9	22	10	59
4	187	19	25	88	660
5	65	11	23	20	41
6	40	10	56	26	76
7	80	22	150	9	175
8	89	18	65	8	48
		P Value [e] = 0.0117		P Value = 0.0117	

a. PHA induced proliferation calculated from [^{3}H] thymidine incorporation using the formula B-A/A x 100 where A= baseline proliferation of patient's PBMC measured by day 1 post injury and B= proliferation of PBMC of the same patient corresponding to the day of assessment of T cell proliferation and IL-10 production.

b. T cell proliferation was assessed in a [^{3}H] thymidine incorporation assay by culture of monocyte-depleted SRBC-rosetted T cells ($2x10^5$cells/200µl/well) in presence of immobilized anti-CD3 (1.5µg/well) plus anti-CD4 (1µg/well) for 72 hours and expressed as DPM.

c. IL-10 levels in the supernates of T cells cultured in presence of immobilized anti-CD3 (1.5µg/well) plus anti-CD4 (1µg/well) for 24 hours, were measured by ELISA.

d. 8 samples were collected from 3 patients at different post injury days.

e. Statistical significance (P) between normal and patient values were determined by Wilcoxon non-parametric test.

Table 13 Depressed T cell IL-10 production in trauma patients also having depressed T cell proliferation [a]

Experiment	% Decrease T cell proliferation	IL-10 [b] (pg/10^6 T cells/ml) Normal	Patient [c]
1	99	39	<5
2	97	117	<5
3	83	117	37
4	94	64	<5
5	60	108	<5
6	31	100	37
7	32	53	<5
8	56	156	<5
9	54	63	<5
		P Value [d] =	0.007

a. T cell proliferation was assessed in a [^{3}H] thymidine incorporation assay by culture of monocyte-depleted SRBC-rosetted T cells ($2x10^5$cells/200µl/well) in presence of immobilized anti-CD3 (1.5µg/well) plus anti-CD4 (1µg/well) for 72 hours. When the proliferation of T cells from the patient is decreased >30% as compared to the paired normal, it is considered depressed.

b. IL-10 levels in the supernates of T cells cultured in presence of immobilized anti-CD3(1.5µg/well) plus anti-CD4 (1µg/well) for 24 hours, were measured by ELISA.

c. 9 samples were collected from 4 patients at different post injury days.

d. Statistical significance (P) between normal and patient values were determined by Wilcoxon non-parametric test.

Table 14 Trauma patients having elevated MØ TNFα levels also have depressed MØ IL-10 levels

	MØ TNF_α [a] (ng/10^6MØ/ml)		MDP Induced MØ IL-10 [b] (ng/10^6MØ/ml)	
Experiment	Normal	Patient [c]	Normal	Patient
1.	0.847	5.755	2.094	0.728
2.	1.154	11.603	5.660	0.393
3.	< 0.05	7.262	1.139	< 0.005
4.	8.342	68.875	2.842	0.348
5.	8.103	28.586	2.555	< 0.005
6.	< 0.05	4.813	10.466	0.461
7.	4.915	10.167	2.216	0.493
8.	6.125	12.372	6.964	0.704
9.	< 0.05	24.224	5.944	0.755
	P Value [d] = 0.0077		P Value = 0.0077	

a. TNF_α levels, cell-associated (MØ Lysate) plus secreted (MØ Supernates), of MØ cultured in medium and MDP (20 μg/ml) for 16 hours, were assessed by LM bioassays.

b. IL-10 levels in the supernates of MØ cultured in medium and MDP (20 μg/ml) for 16 hours, were measured by ELISA.

c. 9 samples were collected from 7 patients at different post injury days.

d. Statistical Significance (P) between normal and patient values determined by Wilcoxon non-parametric test.

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